Supporting Information

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SI Materials and Methods

Animals and Injections. Animal experiments were approved by the Institutional Animal Care and Use Committee and conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and the recommendations of the International Association for the Study of Pain. Animals were housed 2–5 per cage and maintained on a 12-h light/dark schedule in a temperature-controlled environment with ad lib access to food and water. DTX (100 μg/kg) (List Biological Laboratories) was injected i.p. on 2 days, separated by 72 h. Behavioral tests were performed 7 to 31 days after the first DTX injection. For intrathecal capsaicin studies, adult male C57Bl6 mice (20–30 g; Charles River) were anesthetized with 1.5% isoflurane (vol/vol) and injected intrathecally with capsaicin (10 μg) or vehicle (10% ethanol (vol/vol), 10% Tween 80, saline (vol/vol)) in a volume of 5.0 μL with a luer-tipped Hamilton syringe at the level of the pelvic girdle. Behavioral tests were performed 1 to 16 days after capsaicin injection.

Generation of Mrgprd^{DTR} Mice. A targeting construct composed of DTR followed by encephalomyocarditis virus internal ribosomal entry sites (EMCV-IRES), EGFPf, and a self-excising *loxP*-flanked neomycin-resistant cassette (ACN) (1) was designed to replace the entire coding region of *Mrgprd*, located within the second exon. To clone DTR into the ATG initiation site, 2 separate segments were created by PCR with overlapping oligonucleotide pairs. The overlapping sequences, 5′-ggt tct tct tct acc cat agg aCC ATG GGC ATG AAG CTG CTG CCG TCG GTG G-3′, contain the 5′ untranslated sequences of *Mrgprd* (50 bp upstream from the ATG initiation codon, small letters) and DTR sequences fused together. The PCR products of the 2 segments were gel-purified and used as templates for the final PCR using the 5′ primer of the first segment and 3′ primer of the second segment, which contain the entire coding sequences of DTR. These 2 primers contain restriction sites allowing the PCR product to be cloned into the Mrgprd targeting construct as described previously (2). Gene targeting in embryonic stem cells by homologous recombination was performed as described (2).

Histology. Immunohistochemistry (IHC) was performed on spinal cord, DRG, and skin as previously described (2–4). Antibodies used were as follows: rabbit anti-GFP (1:1,000; Molecular Probes), chicken anti-GFP (1:1,000; Aves Labs), goat anti-DTR (1:500; anti-human HB-EGF; R&D), rabbit anti-5HT-1D (1:10,000; gift of A. Ahn, San Francisco, CA), rabbit anti-AQP1 (1:10,000; Chemicon, Inc.), mouse anti-calbindin (1:5,000; Sigma), guinea pig anti-CGRP (1:800; Peninsula), rabbit anti-CGRP (1:100,000; Peninsula), rabbit anti-NK1 receptor (1:4,000; Novus Biologicals), guinea pig anti-TRPV1 [1:5,000; diaminobenzidine (DAB) or 1:1,000 (fluorescence); gift of D. Julius, San Francisco, CA], rabbit anti-TRPV2 (1:15,000; gift of D. Julius, San Francisco, CA), mouse anti-NeuN (1:1,000; Chemicon), and rabbit anti-Fos (1:50,000; Oncogene). For fluorescent IB4 binding, we included 1:100 isolection GS-IB4 (either Alexa Fluor 647 or Alexa Fluor 568 conjugated; Molecular Probes) during the secondary antibody incubations. For DAB-labeled IB4 binding, biotinylated IB4 (1:500; Vector Laboratories) was added instead of primary antibody solution.

Fos Expression. Three mice per treatment group were anesthetized with 1.5 g/kg i.p. urethane (Sigma) 20 min before immersion of the hind paw in a 55 °C water bath, up to the ankle, 3 times for 30 s each time, with a 1-min interstimulus interval. Two hours after the stimulus, mice were perfused, and L4/L5 spinal cord sections were processed for Fos IHC. Digitized images of 10 randomly selected sections per animal were captured using both bright-field and darkfield illumination. Using the darkfield image as a guide, a line was drawn at the border between the relatively dark substantia gelatinosa (lamina II) and the more lucent or opaque lamina III. Counts of Fos immunoreactive profiles were taken for lamina I/II and V.

Densitometry. Digitized images were captured with a CCD camera (Zeiss Axiocam) attached to a Nikon Eclipse microscope. Image analysis was performed using NIH Image software (http://rsb.info.nih.gov/nih-image). Briefly, the area of staining was outlined, and pixel density within the selected area was then measured and multiplied by the total area selected. Data were collected from 5 randomly selected sections from at least 4 animals per treatment group.

Fluorogold Injections. One week after capsaicin or vehicle treatment, 3 animals from each group were used for a Fluorogold (Fluorochrome, LLC) retrograde tracing study. Animals were anesthetized with a mixture of ketamine and xylazine (60 mg/kg and 8 mg/kg, respectively), a laminectomy was made to expose the L4 or L5 segment of the spinal cord, and the dura was then retracted. Three separate 1.0-μL injections of Fluorogold were made in each side of the spinal cord, ≈1.0 mm apart in the rostrocaudal direction, at a depth of ≈2.0 mm from the dorsal surface of the spinal cord. A small piece of Gelfoam (Cardinal Health) was soaked in saline and placed over the laminectomy site, and the incision was then closed with 6–0 silk suture (Ethicon). Animals were perfused after a survival time of 2 days, and DRG tissue was processed for TRPV1 IHC. For cell counts, at least 500 total NeuN$^+$ cells were counted per animal, and the number of TRPV1$^+$ cells was determined as a percentage of NeuN$^+$.

Behavior. All behavioral testing was performed blind to genotype and treatment group during the animals' light period. WT and Mrgprd^{DTR} mice were individually housed at least 1 week before behavioral tests. Where applicable, animals were habituated to the test apparatus for 60 min before testing.

von Frey Test of Mechanical Threshold. Mice were placed in plastic chambers on a wire mesh grid, and the plantar surface of the hind paw was stimulated with von Frey filaments (Stoelting Co. or North Coast Medical, Inc.) according to the up-down method (5).

For response frequency measurements, a series of von Frey filaments of increasing stiffness (0.16, 0.4, 0.6, 1.0, and 1.4 g) were applied. Each filament was applied 10 times with at least a 1-min interval between 2 consecutive stimuli to the same hind paw. Results are expressed as the average percentage of withdrawal responses for a given stimulus force value.

Tail Immersion Test. The tail immersion test was performed as described previously (6). Cutoff times were 60 s (at 44 °C) and 30 s (at 46, 48, 50, and 52 °C), after which the tail was removed from the bath regardless of response.

Hot Plate Test. For Mrgprd^{DTR} mice, animals were placed on a metal plate (IITC; Life Science) preheated to 48, 50, 52 or 55 °C,

and latency to hind paw licking, shaking, or jumping was measured as the average of 3 trials per mouse taken ≥5 min apart. Cutoff latencies were 80 s (at 48 °C), 40 s (at 50 °C), and 20 s (at 52 and 55 °C).

For capsaicin-treated mice, animals were placed on a hot plate apparatus (Columbus Instruments), and latency to lick the hind paws or jump was recorded as the average of 3 trials per animal taken ≥5 min apart. Cutoff latency to avoid tissue damage was 30 s.

Hargreaves (Radiant Heat) Test. Animals were placed in plastic chambers on a glass surface through which radiant heat was focused on the hindpaw (Radiant heat apparatus supplied by Department of Anesthesiology, University of California San Diego, La Jolla, CA for intrathecal capsaicin studies and IITC for *Mrgprd*^{DTR} studies). Latency to withdraw the paw was measured as the average of at least 3 trials per animal taken ≥5 min apart. Cutoff latency to avoid tissue damage was 1 min.

Single Cold Plate Test. Mice were placed on an aluminum plate cooled to −5 °C. Latency to display a vigorous withdrawal of the hind paw was measured as the average of 2 trials per animal taken ≥5 min apart. Cutoff latency to avoid tissue damage was 1 min.

Temperature Preference. A 2-plate choice task was used to assess temperature preference. Animals were placed into a plastic chamber whose floor consisted of 2 identical aluminum plates, the temperature of which could be regulated independently. The time the animal spent on each of the 2 sides was measured during 5 min. A trial was aborted if the mouse failed to at least touch both plates within the first minute. To control for possible side preference or room cues, the test was repeated once with the location of the plates switched. Mice were first acclimated to the 2-plate apparatus with a trial in which both plates were set to 32 °C (for *Mrgprd*^{DTR} mice) or 30 °C (for capsaicin-treated mice). On subsequent days, the temperature of one plate was set to a range of temperatures from 5–45 °C, whereas the other was held constant at 32 or 30 °C, and time spent on the 32 or 30 °C plate was measured as an average of the 2 trials.

CFA-Induced Heat and Mechanical Hypersensitivity. An intraplantar injection of 20 μL of undiluted CFA (Sigma) (Figs. 1 *C* and *D* and 3 *C* and *D*) or 10 μL of a 1:1 saline/CFA (Figs. 5*C* and 6*B*) emulsion was made through a 30-gauge needle. Heat and mechanical thresholds were measured using the hind paw radiant heat and von Frey tests, as described previously, beginning 24 h after the injection and then daily for 1 week.

Statistical Analysis. Behavioral, densitometry, and Fos data were analyzed by the Student's *t* test, one- and two-way repeated measures ANOVA (Bonferroni posttest), or the Mann-Whitney *U* test, with $P < 0.05$ considered to be significant.

1. Bunting M, Bernstein KE, Greer JM, Capecchi MR, Thomas KR (1999) Targeting genes for self-excision in the germ line. *Genes Dev* 13:1524–1528.
2. Zylka MJ, Rice FL, Anderson DJ (2005) Topographically distinct epidermal nociceptive circuits revealed by axonal tracers targeted to Mrgprd. *Neuron* 45:17–25.
3. Lewinter RD, Skinner K, Julius D, Basbaum AI (2004) Immunoreactive TRPV-2 (VRL-1), a capsaicin receptor homolog, in the spinal cord of the rat. *J Comp Neurol* 470:400–408.
4. Shields SD, Mazario J, Skinner K, Basbaum AI (2007) Anatomical and functional analysis of aquaporin 1, a water channel in primary afferent neurons. *Pain* 131:8–20.
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6. Lee H, Iida T, Mizuno A, Suzuki M, Caterina MJ (2005) Altered thermal selection behavior in mice lacking transient receptor potential vanilloid 4. *J Neurosci* 25:1304–1310.

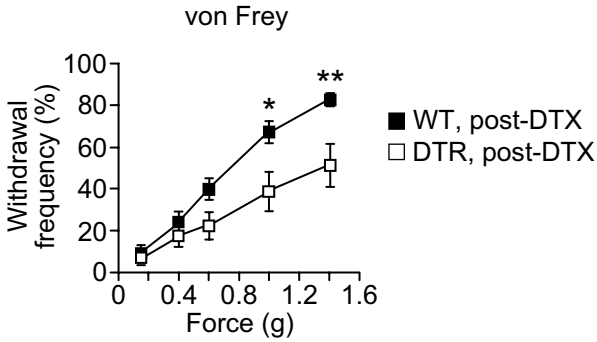

Fig. S1. Mice lacking Mrgprd^{+} neurons exhibit deficits in mechanosensitivity. Paw withdrawal frequency to von Frey filaments post-DTX ($n = 8$ per genotype; *, $P < 0.05$, **, $P < 0.01$, two-way ANOVA with Bonferroni posttests). Data represent mean ± SEM.

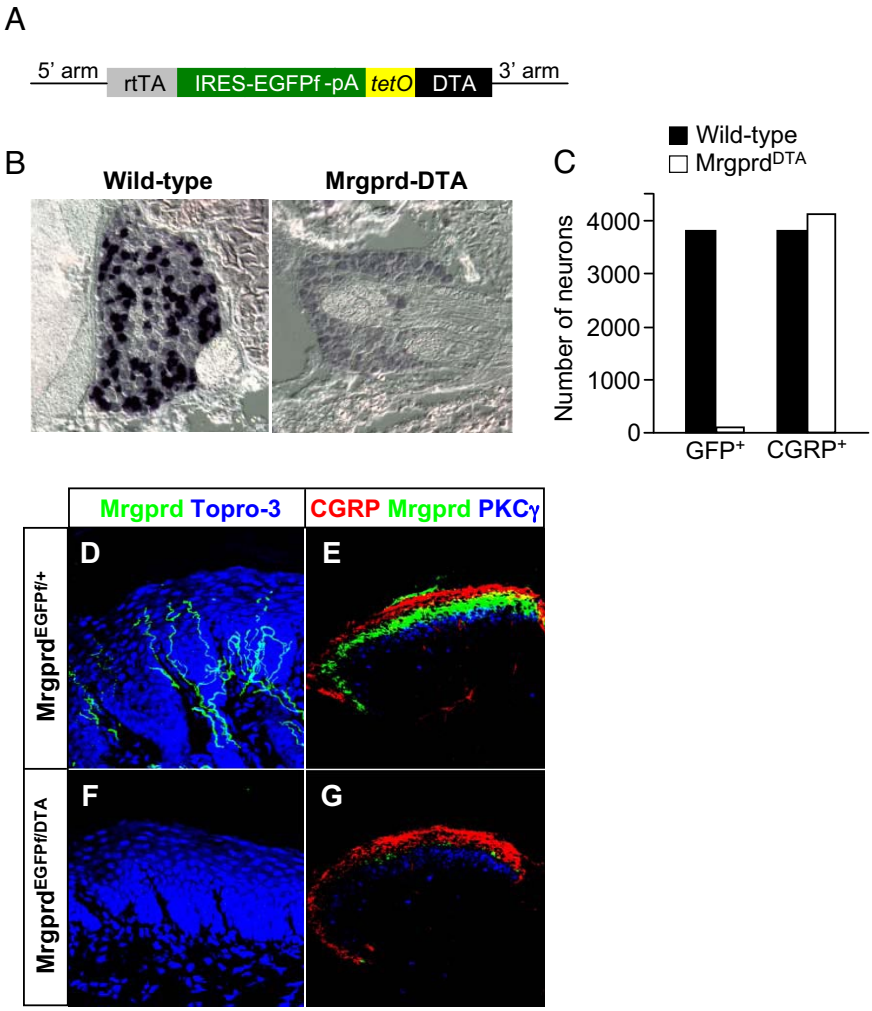

Fig. S2. Mrgprd$^+$ neurons are selectively ablated in *Mrgprd*^{DTA} mice. (*A*) The targeting construct was designed to express diphtheria toxin A fragment (DTA) in Mrgprd$^+$ cells in the presence of doxycycline. However, *Mrgprd*^{DTA} mice expressed DTA in a doxycycline-independent manner, causing an ablation of Mrgprd$^+$ neurons from birth. (*B*) In situ hybridization was performed with a *Mrgprd* antisense riboprobe on adult DRG sections from WT and *Mrgprd*^{DTA} mice. (*C*) The total numbers of GFP$^+$ and CGRP$^+$ L5 DRG neurons in WT and *Mrgprd*^{DTA} mice ($n = 1$ each) are shown. Note selective and complete loss of *Mrgprd*$^+$ cell bodies. (*D*) Sections of the glabrous skin (*D* and *F*) and spinal cord (*E* and *G*) from *Mrgprd*$^{EGFPf/+}$ mice (*D* and *E*) and *Mrgprd*$^{EGFPf/DTA}$ mice (*F* and *G*) were stained for the indicated markers. (*F* and *G*) Note selective loss of Mrgprd$^+$ fibers (green).

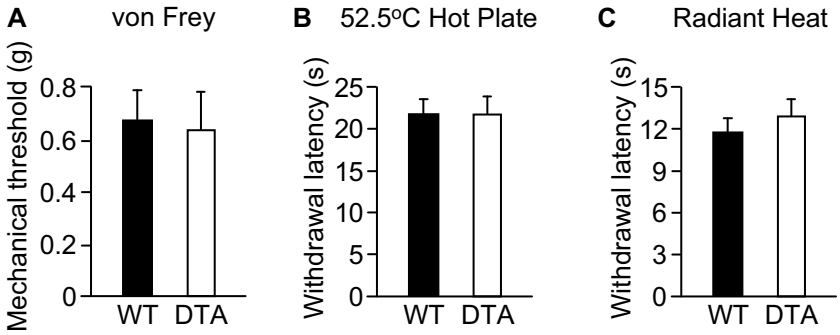

Fig. S3. Mice lacking Mrgprd^{+} neurons from birth exhibit normal mechanical and thermal sensitivity. von Frey (*A*), hot plate (*C*), and radiant heat (*D*) tests [n = 9 for WT and n = 8 for *Mrgprd*^{DTA} (''DTA'') mice]. Data represent mean ± SEM.

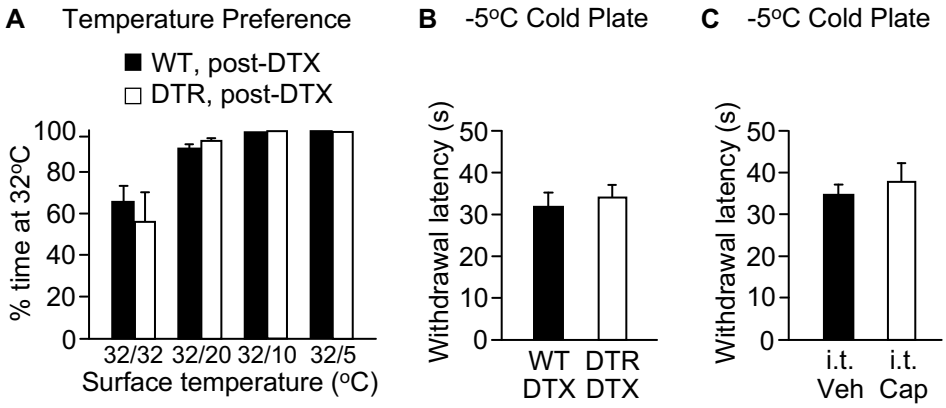

Fig. S4. Mice lacking Mrgprd^{+} neurons and capsaicin-treated mice exhibit normal cold sensitivity. (*A*) Percentage of time spent at 32 °C during a 5-min test period when given a choice between 2 temperatures (32 vs. 32 °C, 32 vs. 20 °C, 32 vs. 10 °C, 32 vs. 5 °C; $n = 10$ for all groups). (*B* and *C*) Paw withdrawal latency on a −5 °C plate [$n = 11$ for WT/DTX mice vs. $n = 9$ for *Mrgprd*^{DTR}/DTX mice and $n = 10$ for intrathecal (i.t.) vehicle vs. $n = 8$ for i.t. capsaicin]. Data represent mean ± SEM.

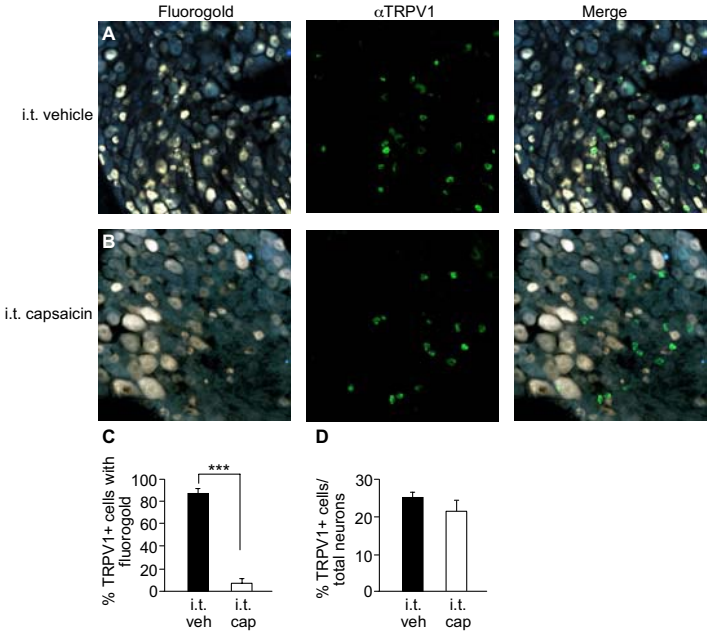

Fig. S5. Intrathecal capsaicin destroys the central terminals but not the DRG cell bodies of TRPV1$^+$ afferents. One week after injection of intrathecal capsaicin or vehicle, mice received a lumbar spinal injection of the retrograde tracer, Fluorogold. (*A*) In L4 or L5 DRG of vehicle-treated mice, the vast majority of TRPV1$^+$ cells (*Center*) contain Fluorogold (*Left*). (*B*) In capsaicin-treated mice, very few TRPV1$^+$ cells (*Center*) contain Fluorogold (*Left*). Right panels show merged images. (*C*) % of TRPV1$^+$ cells that contain Fluorogold. (*D*) TRPV1$^+$ DRG neurons, expressed as a percentage of NeuN$^+$ cells, demonstrating that capsaicin treatment did not alter TRPV1 staining of DRG cell bodies. Data presented as mean ± SEM (***, $P < 0.0001$, Student's *t* test; $n = 3$ for all).

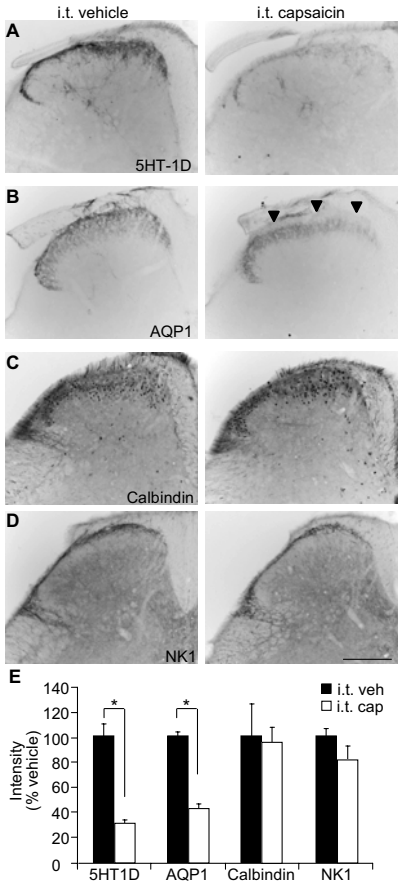

Fig. S6. Intrathecal capsaicin selectively reduces immunostaining for markers of TRPV1$^+$ afferents. Immunostaining of lumbar dorsal horn 16 days after injection of intrathecal vehicle (*Left*) or capsaicin (*Right*). 5HT-1D receptor (*A*) and aquaporin-1 (AQP1) (*B*) immunoreactivity was significantly reduced by capsaicin. Note that staining for AQP1, which is found in both TRPV1$^+$ and TRPV1$^-$ nociceptors, was depleted in laminae I and outer II (arrowheads), namely, in the region targeted by the TRPV1$^+$ afferents but not in inner lamina II, which is targeted by Mrgprd$^+$ afferents. Staining for calbindin (*C*) and substance P receptor (NK1) (*D*) did not change. (*E*) Density of staining for the different markers Data presented as mean $\pm$ SEM (*, $P < 0.005$, Student's *t* test; for NK1 staining, $n = 6$ per group and $n = 4$ per group for all others.) (Scale bar: 200 μm.)

Table S1. Selective elimination of IB4$^+$ neurons in *Mrgprd^{DTR}*/DTX mice

	Mrgprd$^{EGFPf/+}$	*Mrgprd$^{EGFPf/DTR}$*
% IB4$^+$ neurons†	33.5 ± 1.5%	5.9 ± 0.5%*
% Mrgprd$^+$ neurons†	29.4 ± 2.6%	0.5 ± 0.5%*
% CGRP$^+$ neurons‡	27.4 ± 3.5%	41.0 ± 7.4%*
% Mrgprd$^+$ neurons‡	31.3 ± 3.7%	0.4 ± 0.5%*
% CGRP$^+$ neurons (predicted)§		39.9%

DTX injection in adult *Mrgprd^{DTR}* mice eliminates IB4$^+$ but not CGRP$^+$ DRG neurons. Five control (*Mrgprd^{EGFPf}*) and 4 *Mrgprd^{DTR}* mice, aged 8–10 weeks old, were injected with DTX (100 μg/kg) at day 1 and day 4 and then killed between 2 and 4 weeks after the second injection. At least 4 to 9 DRG from each perfused animal were collected and sectioned. After triple labeling with IB4, GFP and NeuN, or CGRP, GFP, and NeuN, sections from the different genotypes were imaged by confocal fluorescence microscopy using the same gain settings. Marker-positive cells were counted in the different color channels using Adobe Photoshop. The percentages of IB4$^+$, Mrgprd$^+$ (GFP$^+$), or CGRP$^+$ neurons were determined by dividing the total number of marker-positive cells by the total number of NeuN$^+$ cells in each section. Ten to 20 sections were counted for each marker for each animal. Data represent mean ± SD. *Mrgprd^{DTR}* mice without DTX injection have a similar % of IB4$^+$ and CGRP$^+$ neurons as control mice injected with DTX (data not shown). About 4.9 ± 2.8% of CGRP$^+$ neurons are also GFP$^+$, and about 3.8 ± 1.7% of GFP$^+$ neurons are also CGRP$^+$. On average, about 1.2 ± 0.7% of NeuN$^+$ cells are double-positive for CGRP and GFP. *$P < 0.0001$ between the same marker expressed in animals with a different genotype. †Pairwise comparison on the same sections double-stained for IB4 and Mrgprd. ‡Pairwise comparison on the same sections double-stained for CGRP and Mrgprd. §"% CGRP$^+$ neurons (predicted)" indicates the predicted percentage of CGRP/NeuN$^+$ neurons, assuming that all Mrgprd$^+$ neurons, and only those neurons, are ablated. The number obtained (39.9% of CGRP$^+$ neurons) is similar to the measured % of CGRP$^+$ neurons (41%) after DTX administration. We conclude that there is no significant reduction in the CGRP population after DTX administration.